PCT

(51) International Patent Classification 5 :		(11) International Publication Number:	WO 94/13835
C12Q 1/68, 1/00, G01N 33/543	A1	(43) International Publication Date:	23 June 1994 (23.06.94)

(21) International Application Number: PCT/US93/12307

(22) International Filing Date: 16 December 1993 (16.12.93)

(30) Priority Data:
9226229.4   16 December 1992 (16.12.92)   GB

(71) Applicant (for all designated States except US): GRANTA LABORATORIES LIMITED [GB/GB]; 123 Cambridge Science Park, Mitlon Road, Cambridge CB4 4FZ (GB).

(72) Inventors; and
(75) Inventors/Applicants (for US only): BOGDANSKI, Stanley, L. [US/GB]; 6 Meadow Brow, Alderley Edge, Cheshire SK9 7XD (GB). NOWINSKI, Stefan [GB/GB]; 1 Selwyn Road, Cambridge CB3 9EA (GB).

(74) Agent: WILLIAMS, John, N.; Fish & Richardson, 225 Franklin Street, Boston, MA 02110 (US).

(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
    With international search report.

(54) Title: DETECTION OF MACROMOLECULES

(57) Abstract

The invention features a method and system for the detection of macromolecules. The system includes a light source, a probe comprising a former of predetermined dimensions, and a detector, all arranged in a geometry such that the detector observers the diffraction pattern of the probe. The former has an active surface of such dimension that its diffraction pattern at the detection plane is of the order of centimeters from first to second order peaks, whereby changes on the active surface of the order of nanometers in the dimension result in movements of the order of millimeters in the position of the diffraction peaks allowing the attachment of macromolecules to the active surface of the probe to be detected. The invention is also useful for detecting biological macromolecules, having clinical significance for diagnosis, of disease, directly without the use of complicated labelling procedures as used in some other diagnostic techniques. The probe can be also used in conjunction with other known techniques since the active surface is able to highly concentrate the macromolecules relative to their concentration in the solution.

DETECTION OF MACROMOLECULES

This invention relates to the detection of macromolecules.

5   There are many highly selective and sensitive analytical methods for the detection of macromolecules of a biological interest. The selectivity of the method, in many cases, is based on the complementary structure which exists between certain pairs of molecules, such as:

10   a DNA or RNA single strand molecule and its complementary single strand,

an antibody and its antigen, and

an enzyme and its substrate (or coenzyme).

Essentially, it is these molecular interactions
15 which give the analyses the ability to determine the presence of one particular molecule, the analyte, amidst many other compounds of a similar type. The reagent used comprises the molecular fragment which associates itself to the analyte.

20   Enhanced sensitivity and an improved limit of detection is often achieved by use of a reagent labelled with a molecular tag. The label makes the reagent and, therefore, the analyte, highly detectable by rendering it fluorescent, bioluminescent, chemiluminescent,
25 radioluminescent, colourimetric, etc. A further technique for labelling, is by attaching an enzyme to the reagent and measuring the changes brought on by the introduction of the appropriate substrate.

Although detection limits as low as 600 molecules
30 of analyte have been reported, immunoassays which are normally used can detect down to 10^{-14} mol/l of analyte, which is sufficient for many applications involving drugs, metabolites or gene products. Infection and infectious agents, as well as food contamination are two
35 areas which benefit from the lowering of the limit of detection.

One way of improving the detection limit is to increase the number of analyte molecules in the sample. Culturing samples is the most common approach at this time, but the use of polymerase chain reaction (PCR) and
5 similar techniques applicable to DNA are expanding. Amplification techniques often result in multiplying the interfering substances along with the analyte.

Assay formats are classed as either heterogeneous or homogeneous, depending on whether a reagent is
10 immobilized on a solid or whether it remains in a liquid, respectively. Assays are also classified as to whether they are competitive or non-competitive.

In a competitive, heterogeneous assay, there are two reagents. The first, R1 is selective towards the
15 analyte, A, and is immobilized say on the surface of a microtitre plate. The sample containing the analyte is introduced and then washed away, leaving some of the R1 occupied by the analyte. A second reagent, R2 which is labelled and selective for the first reagent, R1, is
20 added and washed away. The response of the detector towards the labelled reagent is indirectly proportional to the amount of analyte originally present.

In a non-competitive, heterogeneous assay two reagents are used. The first is immobilized and
25 selective to the analyte. The analyte is captured, as described above. The second regent is labelled and selective to the analyte. The following detector signal is directly proportional to the concentration of the analyte.

30 Homogeneous techniques basically are the same as the above but rely on a measurable change occurring to labelled reagent when it becomes associated to an analyte. Homogeneous assays are mostly of the competitive type and are usually relatively simple to

- 3 -

perform and automate but have limited sensitivity, i.e.
10^{-9} mol/l.

These assay formats which involve multiple steps,
having two or more reagents, make the analysis more
5 complex and more prone to errors and contamination. The
use of labels on reagents often indicates another level
of conditions must apply which may compromise those which
result in the best reagent selectivity and
reagent/analyte affinity. The labels and their detection
10 are subject to a range of potential interferences, which
lead to inaccurate interpretation of the final results
unless procedures are carefully followed in a clean
environment. Because of these difficulties, many of the
analytical methods which are developed within the
15 research laboratory will never be used in general
practice. Direct detection of the analyte or
analyte/reagent offers the best means of reducing
potential interferences and analysis complexity.

A range of new direct measurement techniques have
20 recently been reported which are being developed by major
companies. One company has recently launched an
analytical instrument based on Surface Plasmon Resonance
and others are developing techniques using the
ellipsometric and interferometric detection of an
25 evanescent wave. Other investigations are looking at the
changes of the oscillation frequency of a piezo electric
crystal and the alteration to a surface acoustic wave due
to the presence of an associated analyte. In each of the
above methods, the reagent is immobilized on the surface
30 of interest. Although still in their infancy, these new
techniques appear to be inherently expensive and lack
sensitivity.

The present invention is relatively simple to
implement, has great sensitivity and retains the
35 advantage of a direct analytical measurement.

- 4 -

We describe below a heterogeneous method in which the unlabelled reagent molecule is attached to a surface at the edge of a small former which is configured so as to create a diffraction pattern when placed into a
5 specially designed detector. Changes in this diffraction pattern indicate whether the reagent is associated with an analyte or whether it is unassociated, i.e., no analyte is present. The former can be configured in several ways to achieve an amplified diffraction pattern
10 and allow an analyte measurement, e.g., as a slit, as a wire, as a ball or as multiples of these discrete elements.

Our method is based on the spatial redistribution of the diffracted light leaving a discrete element or
15 former of small dimension which results from the apparent change in dimension of that former when it becomes associated with analyte molecules. The active surface where the molecular association takes place lies along the path of the light used to illuminate the former and
20 forms the edge of the former thus defining the dimension producing the diffraction pattern. For a given application and a specific former and analyte molecule both the spatial distribution of the diffraction pattern and the change in it which occurs due to the presence of
25 the analyte are well defined and are used to advantage in a system which is highly selective for the analyte and minimizes interferences.

The advantages derived from using a discrete former element rather than a multiplicity of features
30 that cooperate to form a diffraction pattern, such as in a grating, are several. The relatively simple diffraction pattern produced by a discrete element is more easily interpreted and less subject to external changes. Uniformity from element to element within a

- 5 -

pattern is not an issue. The smaller surface area of a discrete element results in a lower limit of detection for the analyte.

Our method allows detection of the analyte
5 directly, without the necessity of further amplification, e.g., by PCR (polymerase chain reaction), or the use of labelled reagents. This results in a direct analysis, less prone to interferences and contamination, optimized for selectivity, and offering a relatively simple
10 analytical procedure. The technique is amenable to the use of reagent labels and additional reagents to further improve the sensitivity and limit of detection.

The probe concentrates the analyte from a relatively large volume of solution to a known specific
15 small area, depending on its binding affinity between captured reagent on the probe and the analyte. This enhances sensitivity and lowers the level of detection as compared to optical techniques that depend on the analyte concentration on a larger surface area or in a larger
20 volume, such as those depending on the reduction of light being transmitted. We envision the use of detection systems other than diffraction, which take advantage of the enhanced signal resulting from this concentration step and localization of the analyte.

25 The detection technique includes multiple analyte detection, blank readings and tests for false positives and false negatives by incorporating the appropriate sections into the probe during the fabrication. This is accomplished without increasing the complexity of the
30 analytical procedure.

In certain embodiments, the probe is configured to perform as a continuous detector, say on the eluent of a chromatographic column, or to serve as a consumable, for a preliminary, quick test to be performed by a doctor in
35 his surgery. The probe giving a positive test result and

- 6 -

which is presumed to contain the analyte can be sent to a
clinical laboratory for further tests, including
amplification, and final confirmation.

Accordingly, in one aspect, the invention features
5 method and a system for detection of macromolecules that
employs a probe comprising a discrete former of
predetermined small size and selected geometry capable of
diffracting light of a selected wavelength to produce a
diffraction pattern, the former having an active surface
10 which lies along the path of the light with reagent
molecules exposed for binding the macromolecules to the
active surface; a light source adapted to emit light of
the selected wavelength, the radiation being directed to
and extending along the active surface; the wavelength of
15 the light and the small size of probe being so related
that the macromolecules when bound to the active surface
are non-transparent and produce a diffraction pattern
change which is detectable and greatly amplified in
dimension relative to the size of the macromolecule that
20 produces the change; a detector arranged to detect at
least a portion the diffraction pattern; and a system
control adapted to determine the presence of the
macromolecules on the basis of the change of the
diffraction pattern.

25   Preferred embodiments of this aspect of the
invention may have one or more of the following features.

The limited size of the active surface of the
probe is effective to concentrate the macromolecules of
interest on its surface for detection.

30   The reagent molecules are either DNA or RNA
molecules of a sequence complementary to the sequence of
DNA or RNA molecules of interest, or protein molecules.

The protein molecules are either enzymes adapted
to detect substrates or co-enzymes, or antibodies
35 associated to an antigen of interest.

The critical dimension of the probes' active surface is of the order of 0.1 to 20 microns and the macromolecules being detected have dimension of the order of fractions of a nanometer to nanometers.

5   The probe has a wire former that includes an intermediate layer with attached reagent molecules.

The probe comprises a former that defines a slit further including an intermediate layer with attached reagent molecules at the edges of the slit.

10   The probe comprises a 0.1 to 20 micron size sphere former that includes an intermediate layer with attached reagent molecules.

The probe comprises a former that defines an aperture including an intermediate layer with attached
15 reagent molecules at the edge of the aperture.

The reagent molecules are arranged in a manner to form detection and reference surfaces.

The detection and reference surfaces enable quantitative or qualitative detection of the
20 macromolecules.

In another aspect the invention features an apparatus for the detection of macromolecules comprising a light source, a probe comprising a former of predetermined dimensions and a detector, all arranged in
25 a geometry such that the detector observes the diffraction pattern of the probe, the former having an active surface of such dimension that its diffraction pattern at the detection plane is of the order of centimeters from 1st to 2nd order peaks, whereby changes
30 on the active surface of the order of nanometers in the dimension of the probe result in movements of the order of millimeters in the position of the diffraction peaks allowing the attachment of macromolecules to the active surface of the probe to be detected.

- 8 -

Preferred embodiments of this aspect of the invention may have one or more of the following features.

The radiation is modified (such as being polarized), to enhance the interaction of the radiation 5 with the analyte macromolecules. The radiation is parallel and coherent to generate the optimum diffraction pattern.

The macromolecules are modified by staining or other process. The macromolecules are labeled by a 10 radioactive, fluorescent, bioluminescent, chemiluminescent, electroluminescent, chromophoric or conductive element, or an enzyme.

The radiation making up the diffraction pattern is monochromatic, achieved, e.g., by use of a monochromatic 15 source or a narrow band filter anywhere in the beam path prior to the detector.

The probe is of a "positive" shape such as a wire or a sphere, or a "negative" shape such as a slit or a pin-hole having at least one dimension of a suitably 20 small magnitude so as to create a suitably large diffraction effect that results in magnification of surface changes.

In another aspect, the invention features a system and method of detection of macromolecules (as in 25 diagnosis of disease when the macromolecules are biological molecules of clinical significance) whereby a suitable reagent (biological examples include: single strand DNA, RNA antibodies, or other reagent molecule) is attached to an active surface of small dimensions and the 30 probe is presented to a suitably prepared sample, the presence of analyte molecules in the sample resulting in binding of those molecules to the reagent molecules and producing a small dimensional change on the active surface and thereafter observing the probe, the presence 35 of analyte molecules being thereby detected directly,

- 9 -

without the use of complicated labelling procedures as used in some other diagnostic techniques.

The invention is hereinafter more particularly described by way of example only with reference to the
5 accompanying drawings in which:

Fig. 1 is a diagrammatic view of an embodiment of analyzer system in accordance with the present invention;

Fig. 2 is one preferred embodiment of an analyte selective probe with active surface located at the edges
10 of a slit;

Fig. 2A is the analyte selective probe of Fig. 2 adapted for in situ detection;

Figs. 3A, 3B, 3C, 3D, 3E, 3F, 3G, 3H, and 3I show schematically a method of fabrication of the analyte
15 selective probe of Fig. 2;

Fig. 4 is another preferred embodiment of the analyte selective probe with the active surface on a wire held within a tube which provides a flow through cell configuration for introducing a sample;
20 Figs. 5A, 5B, 5C, and 5D show fabrication of the analyte selective probe of Fig. 4;

Figs. 6, 6A, and 6B show a transmission mode embodiment of the analyzer system of Fig. 1;

Fig. 7 shows a diffraction pattern monitored by a
25 split detector arrangement of the analyzer system;

Fig. 8 shows multiple diffraction patterns formed by several beams and monitored by multiple detectors of the analyzer system;

Figs. 9 and 9A show a diffraction pattern obtained
30 by a random orientation of the active surface, and an annular detector used to detect this pattern, respectively; and

Figs. 10 and 10A show the reflection mode embodiments using a slit probe and a wire probe,
35 respectively.

- 10 -

Referring to Figs. 1 and 2, a system for detecting
macromolecules includes a source 10 emitting
electromagnetic radiation 12 that is directed to a probe
20, containing the sample and held in a holder 19 in line
5 with a detector 100. An active surface 28 of probe 20 is
aligned and interacts with the radiation. The substances
present on active surface 28 alter the outgoing radiation
16 in a predictable manner which is detected by detector
100. The entire operation is controlled by a process
10 control unit 120 that specifies the type of radiation to
be emitted from source 10, controls location of probe 20
and defines the mode of operation and position of
detector 100. A system operator interacts with control
unit 120 via a computer 122 and a display unit 124. A
15 printer/plotter 126 is used to generate a hard copy.
Detection system 100 has several detectors that can be
used simultaneously or interchangeably depending on the
type of the probe used, wavelength of the detection beam
and reference beams, the overall source-probe-detector
20 arrangement and the mode of operation. Depending on the
mode of operation and the wavelength of the source, the
selected detector is a photomultiplier, photodiode, CCD
array, or photoconductive detector. The invention uses
several types of probe 20.
25   Referring in particular to Fig. 2, analyte-
selective probe 20 includes a rigid transparent substrate
22, a thin SiO_2 layer 24, and a nontransparent layer 26.
A thin section of layers 24 and 26 is removed to form a
transparent slit which is 0.1 μm to 20 μm wide and about
30 0.02 mm to 20 mm long. The exposed silicon dioxide
surfaces 28 of the slit are chemically treated to
introduce reactive sites (-NH_2) which are used to
immobilize single strands of DNA having a sequence of
nucleic acids which form matching base pairs with the
35 complementary nucleic acids found on the targeted analyte

- 11 -

DNA molecule. Surface 28 has a well defined depth,
surface roughness, and geometry and specific orientation
with respect to incoming beam 12.

5   The 0.1μm to 20μm wide slit is fabricated by a
series of photolithographic steps. Referring to Fig. 3A,
probe 20 is formed by depositing thin layer 24 of SiO$_2$ by
chemical vapor deposition (CVD), sputtering or other
technique onto transparent substrate 22 (e.g., microscope
slide lightly coated with thin gold layer to make
10   inactive). The thickness of SiO$_2$ layer 24 is selected
depending on the desired depth of active surface area 28.
A photoresist 25 is deposited on top of the silicon
dioxide layer 24. The first photolithographic (Fig. 3B)
step defines the dimensions of the slit on the probe and
15   starts the process of removing the desired section of
SiO$_2$ layer 24 which exposes active surface 28.
Photoresist 25 is exposed and developed to define the
slit. In the area defined by the photolithographic mask
(Fig. 3B), SiO$_2$ layer 24 is removed by anisotropic
20   etching (or by ion milling) through the SiO$_2$ layer (Fig.
3C). Next, photoresist 25 is removed. The
nontransmitting layer 26 that is opaque to radiation of
selected wavelengths (e.g., a reflecting gold layer) is
deposited onto layer 24. In the second step, photoresist
25   25 is again deposited, exposed and developed to define a
slit that coincides with the removed section in the SiO$_2$
layer (Fig. 3E). Etching (or ion milling) is used again
to create a transparent slit in nontransmitting layer 26
(Fig. 3F).

30   Alternatively, only one photolithographic step is
used after deposition of both layers 24 and 26. Here, a
photoresist is deposited on layer 26, exposed and
developed to define the slit. The slit is created by ion
milling or by combination of ion milling and anisotropic
35   etching through layers 24 and 26.

- 12 -

The silicon dioxide surface 28 is activated for the attachment of a reagent molecule. When using single strand DNA fragments as reagent molecules, the silicon dioxide surface is activated using a silylating agent to
5 form activated ester of silica 29, i.e., N-hydroxysuccinimidyl-silica located on surface 28 (Fig. 3G). The desired single strand DNA fragments are synthesized by standard techniques (e.g., using an Applied Biosystems Synthesizer). The fragments have an
10 additional section with an aminoalkyl group at the 5'-end. The 5'-aminoalkyl-DNA 30 is then coupled to an N-hydroxysuccinimidyl ester silica located on the activated edge surface 28 (Fig. 3H). This reaction gives a stable amide linkage between the DNA and the silica, as
15 described by Solomon, et al. in Analytical Biochemistry 203, 58-69 (1992).

Probe 20 contains a long, thin slit that has the interior SiO_2 edges coated with analyte selective reagent e.g., single strand DNA fragments 30, Fig. 3H and Fig. 2,
20 of a particular, known sequence. The sequence is chosen to be complementary to the sequence of the DNA material, analyte of interest, the particular probe is designed to detect. The probe uses different nucleic acid sequences for detection of different analytes of DNA.
25 Next, active surface 28 of probe 20 is contacted with the analyte solution by immersion or introducing the test solution to the probe to bind the analyte DNA material 32, Fig. 3I. Thus Fig. 3I depicts the probe after
30 it has captured the analyte from the sample and the slit has been reduced in width by the buildup of analyte on the active surfaces of the sides of the slit.

The analyte solution is prepared by lysing cells to release DNA material. The double strand DNA is
35 dissociated into single strands by adjusting the

- 13 -

conditions of temperature, salt concentration and/or pH.
The strands are cut into suitable lengths using
restriction enzymes. The analyte DNA molecule, the
target, may be considerably larger than the associated
5   DNA immobilized on the surface of the probe. The analyte
DNA material is bound to the immobilized strands by
controlling the conditions in the micro-environment of
the slit. A washing step is used to remove potential
interference molecules which are less strongly bound. In
10   many cases, the selectivity of the probe is related to
the number of associated base pairs being formed between
the probe DNA and the analyte DNA, whereas the limit of
detection is more closely related to the size of analyte
molecule and depth of the active surface layer containing
15   the probe DNA.

In another embodiment, we use a wire probe, as
shown in Fig. 4. Wire probe 40 is mounted in a capillary
tube 50 using mounting ends 54A and 54B. The sample and
subsequent wash solutions are introduced to the probe via
20   ports 52A and 52B.

Referring to Fig. 5A, wire probe 40 comprises a
former 42 of 0.1 to 20 microns in diameter (e.g.,
tungsten wire). The former is coated with a thin layer
of silicon dioxide 43 introduced by chemical vapor
25   deposition (Fig. 5B). Referring to Fig. 5C, selected
areas of silicon dioxide layer are then activated using a
silylating compound to form an activated ester such as N-
hydroxysuccinimidyl-silica 44. The desired single strand
DNA segments with a DNA sequence complementary to the
30   sequence of a particular analyte are then immobilized on
activated surfaces 44 creating active surfaces 46A and
46B (Fig. 5D) in the same way as described for the slit
probe.

In a similar manner, other sequences of DNA which
35   are targeting other analytes are immobilized on different

- 14 -

segments of the probe by a series of reaction steps which
mask and reveal different portions of the probe. A
multi-segment probe can be fabricated to indicate a false
positive or false negative test result.

5   In another embodiment, the probe is configured to
detect several analyte DNA molecules with a single
exposure to the sample solution. On introduction of a
test solution, all the above surfaces are contacted and
subsequently washed under the same conditions. The

10 system either examines the sections in series using a
single channel detector or uses multi-channel detectors
or scanning detectors. Thus several analytes can be
detected simultaneously by the analyzer system; this is
schematically shown in Fig. 8.

15   Alternatively, slit probe 20 designed for the
multiple analyte detection includes similar sections as
described above for the wire probe.

        The light source 10 (e.g., an excimer laser,
Helium-Neon laser, tunable YAG laser, etc.) emits

20 continuous or pulsed monochromatic, collimated
electromagnetic radiation of a suitable wavelength.
Depending on the reagent and analyte being detected, the
source is selected on the basis of wavelength from a
range of ultra-violet, visible or infra-red lasers.

25 Alternatively, light source 10 includes a source of white
light. Suitable optics and filters are used to scan the
wavelengths and direct the beam. The radiation is split
into one or more detection beams and one or more
reference beams. The geometry of the beam:active-surface

30 interaction is controlled. Material 26 surrounding the
active surface of probe 20 is opaque to the radiation so
that the plane of the active surface lies in the
direction along the path of the radiation.

        The wavelengths are selected on the basis of the
35 active surface and probe configuration, spectral

- 15 -

absorbance, reflectance or scatter characteristics of the reagent and analyte (suitable stains or other absorption enhancing techniques may be used) and the dimensional change as related to the presence of the analyte. The
5 dimensional change, from fractions of a nm to few microns, is measured by monitoring the corresponding change in the diffraction pattern of the active surface with and without the analyte. The analyzer system can scan the active surfaces at different wavelengths and
10 angles to obtain diffraction patterns with optimal signal-to-noise conditions.

Fig. 6 is a diagrammatic view of the transmission mode embodiment of the slit probe. Source 10 emits collimated monochromatic light 12. Probe 20 is aligned
15 to have plane of the active surface 28 oriented along the path of the radiation. The diffracted light 16 is imaged by a spherical lens 18 onto an image plane 80 at a predetermined distance from the lens. A detector 102 of detection system 100 placed in image plane 80 detects the
20 far field (Fraunhofer) diffraction pattern 90 formed by the beam passing over the slit aperture containing active surface 28. Single element detector 102 scans the image plane and detects intensity and location of each peak of an interference pattern given by:
25
$$\frac{I(\theta)}{I(0)} = (1/\beta \sin \beta)^2$$

wherein θ is the angle from the axis of the straight through beam. Pattern 90 has a series of maxima and minima at distances from the axis of the straight through
30 light which are inversely related to the width of the slit. Thus by detecting the change of spacing of the spatial distribution of the diffraction pattern, with and without analyte, the system detects thickness on the analyte (and other layers) of active surface 28. The
35 output of the detector is digitized and processed in the

- 16 -

data analyzer of computer 122. Fig. 6A shows
diagrammatically wire probe 40 placed in the analyzer
system operating in the transmission mode embodiment.
Fig. 6B shows diagrammatically an embodiment wherein wire
5   probe 40 is used in a flow cell arrangement. In the flow
.cell arrangement, the test solution is recirculated or
added to a flowing stream of cleansing solution.
          Alternatively to scanning the image of the
diffraction pattern, multiple element detector 104,
10   formed by several light detecting elements (e.g.
photodiodes), is located at a fixed position. As shown
in Fig. 7, detector 104 determines changes in the
radiation intensity at a fixed point of a selected peak
of diffraction pattern 90. From the change in the
15   detected intensity, (i.e., movement of the pattern
relative to the detector) presence and concentration of
the analyte is determined. The size of the
photosensitive surface area of the detector is optimized
according to probe dimensions, distance between the
20   detector and probe, and further optical considerations to
give the best analyte resolution. The analyzer system
determines, in real time, changes in the diffraction
pattern that correspond to the active surface changes due
to increasing presence of the analyte.
25   The above described configurations observe changes
on the scale of sub-nanometers to nanometers using a slit
of few microns. This resolution, which is dependent on
the chemical and optical nature of the species present on
active surfaces 28, is sufficient to detect the single
30   strands DNA. Further reagents may be used to enhance the
presence of the analyte and give a large detector
response, e.g., by adding to the dimension of the analyte
layer or making the analyte less transparent to the
radiation from the source.

- 17 -

Referring to Fig. 8, in a multi-beam arrangement, multiple detectors 106, 108, and 110 are used in conjunction with several beams 12 to detect the diffraction patterns from different areas of the wire
5   probe 40. This arrangement can analyze multiple analytes at the same time. Slit probe 20 may comprise several slits with active surfaces 28, each adapted to detect different species of analyte.

The high sensitivity is obtained from the small
10 area of the probe onto which the analyte is concentrated, a high affinity of binding between analyte and immobilized reagent, and a detector based on diffraction, which increases in amplification as the probe size is reduced. (Other direct methods for DNA analysis have
15 signals which are proportional to surface area and, therefore, to number of analyte molecules.) Since a single probe can be fabricated with blank and reference sections as well as containing several captive reagents, a range of internal corrections are possible, e.g., to
20 align the probe and eliminate false readings.

The applications of the probe/detector govern the operating conditions and the design of the probe. A simple "yes/no" qualitative test requiring maximum sensitivity benefits from using a probe with a high
25 captive reagent concentration immobilized on a probe containing a minimum active surface area. The same conditions should be optimized to obtain maximum analyte/reagent affinity. A continuous detector may benefit from a larger active surface area, lower reagent
30 concentration and sub-optimal conditions in order to obtain a quicker response time and avoid interferences accumulating over time.

The analyzer system utilizes one or more detection beams and one or more reference beams arranged to probe
35 different active areas of the probe sequentially or

- 18 -

simultaneously. The concentration of the attached single
strands of DNA reagent is varied along the slit length
periodically or continually. The detection beam may scan
the active surface with varying concentration of the
5   analyte bound to the reagent while the reference beam
scans a corresponding active surface with varying
concentration of the reagent. The signals obtained from
the diffraction pattern are subtracted. The examination
sequences are chosen to calibrate the probe, to eliminate
10 false positive or negative readings, to determine
concentration of one or more analytes in test solution.

          The test solution is brought into contact with the
probe in an open or closed system. Referring to Fig. 2A,
a closed system is achieved by adding a transparent layer
15 27 onto nontransmitting layer 26 to form a closed space.
Then, a suitable micro-flow fluid handling system is
attached to circulate fluid through the closed volume.
The closed system enhances the control over temperature
and pH parameters required to control the binding steps.
20 The wire probe of Fig. 4 is used in the same way, wherein
the closed system is achieved by connection of the micro-
flow system to ports 52A and 52B. During the real time
measurements binding of the analyte is detected by
changes to the time base profile of the detected light.
25   The technique uses either a disposable probe or a
probe that can be regenerated for multiple uses depending
on the application. The multiple use probe is adapted to
release the analyte from the active surface (e.g., by
thermally cycling the probe or by pH cleansing).
30   In another embodiment, the active surface of slit
probe 20 (or wire probe 40) includes multiplicity of
surfaces with single strands of DNA arranged in a
selected geometry that allow only molecules of a certain
length to adhere. For example, there may be two active
35 surfaces separated by a selected distance. Each surface

- 19 -

has an immobilized reagent, and the selection of analyte is based on two different molecular associations having a defined distance (molecular size) between them.

In another transmission embodiment, the source-
5 probe-detector alignment can be simplified by using several randomly positioned wires or micro-spheres as a former. For this arrangement, the diffraction pattern is circular in nature. Referring to Fig. 9, beam 12 irradiates a suspension of spherical probes 60 (e.g.,
10 former is a 3 to 5 micron silica ball similar to the ones used in the solid phase chromatography and creates diffraction pattern 95 that is monitored by a detector 112. Detector 112 has the detector elements arranged in one or more annular rings centered on the straight
15 through beam (Fig. 9A). Detector 112 is positioned at a distance from the axis that enables proper resolution of the diffraction pattern. The greater the distance from the axis, the greater the spread of the diffraction pattern.
20   Figs. 10, and 10A show diagrammatically the reflecting mode embodiment of the analyzer system, wherein the diffraction pattern is observed by detecting the reflected radiation from the probe. Referring to Fig. 10, the reflecting embodiment of slit probe 20
25 requires flat transparent substrate 22 made reflective by depositing an additional reflective layer 23 onto layer 22 prior to the slit formation. Reflective layer 23 reflects the diffracted radiation which is separated from the incoming beam by a half silvered mirror 17 at 45° or
30 a similar optical element suitable for the selected wavelength. Detector system 100 is positioned to observe the reflected diffraction pattern in the same way as discussed above. Similarly, the reflecting embodiment of wire probe 40 (Fig. 10A) includes a mirror surface 41
35 positioned behind the wire. The incoming radiation,

- 20 -

diffracted by the active surface of probe 40, is
reflected back onto the wire and then detected by
detector 100.

Optimization of detection may be achieved by
5 altering the incidence angle of the radiation, by
polarizing the radiation and rotating the polarized plane
angle relative to the active surface, by adjusting the
pulse parameters of radiation, or by modulating the
frequency. Furthermore, the light source can operate in
10 a pulse mode to prevent thermal effects on the active
surface or to scan different regions of the active
surface interchangingly. Each of these techniques
provides a means of internal noise and drift reduction of
the detector as well as that of the environment (e.g.,
15 vacuum, gas or liquid) wherein the measurement is
conducted.

Other probe embodiments use only a former with the
immobilized selective reagent bound directly to the
former. Alternatively, a number of reactive
20 intermediates selectively paired with reactive moieties
can be used.

Reagent molecules can be arranged so as to coil
and grow more dense when affected by the analyte
molecule, effectively changing the active surface.
25 Immobilization occurring at several points between the
reagent and active surface can ensure optimal positioning
of molecules for their subsequent detection. The reagent
molecules so arranged would respond to the angle of the
plane using polarized radiation. This arrangement
30 reduces noise and enhances the detection.

In another embodiment, the SiO_2 layer activated by
a silylating agent is replaced by other "active" layers
to which the preferred type DNA strands are bound, for
example, polystyrene, nitro-cellulose, nylon,
35 polycarbonate or polyurethane. The detection surfaces

- 21 -

are again fabricated by photolithography; however, other
methods such as micromachining, laser ablation or molding
may also be used.

We also envision using probes with different
5 shapes of active surfaces arranged to selectively
concentrate the analyte. An aperture with a 0.1 to 20
micron diameter resolves features down to 0.1 nm layers
of analyte. The slit type apertures were described in
detail; however, other geometries such as circular or
10 square hole apertures may be also used. Furthermore, the
active surfaces may be located on structures that are
complementary to the described apertures. For example,
the probe is an opaque long, thin bar or a dot with the
activated surfaces located on the outside edges. An
15 equivalent of a circular aperture is a small sphere.

Following the introduction of the sample and
association between the probe and analyte, the
diffraction signal can be augmented by enlarging the
analyte layer or making it less transparent. This is
20 accomplished by using reagents which would react or
combine with the now immobilized analyte. These reagents
could merely add to the size of the analyte layer, stain
the analyte, or attach an enzyme (or reactive molecule)
which would subsequently deposit a precipitate in this
25 immediate vicinity. The probe can subsequently be also
reacted with reagents which fluoresce, luminesce, etc.

CLAIMS: - 22 -

    1. A method of detection of macromolecules comprising the steps of:

    (a) providing a probe comprising a discrete element former of predetermined small size and selected
5 geometry capable of diffracting light of a selected wavelength to produce a diffraction pattern, said former having an active surface which lies along the path of the light with reagent molecules exposed for binding the macromolecules to said active surface;

10   (b) the wavelength of the light and the small size of the former being so related that the macromolecules when bound to said active surface are non-transparent and produce a diffraction pattern change which is detectable and greatly amplified in dimension relative to the size
15 of the macro-molecule that produces such change;

    (c) introducing a fluid containing said macromolecules to said active surface to enable binding of the macromolecules;

    (d) directing light of said selected wavelength at
20 said probe to produce said diffraction pattern; and

    (e) detecting and analyzing said diffraction pattern
to detect the presence of the macromolecules on the basis of change in the diffraction pattern.

25   2. A method of detection of macromolecules comprising the steps of:

    (a) providing a light source, a probe comprising a former of predetermined dimensions, and a detector, all arranged in a geometry such that the detector observes a
30 diffraction pattern of the probe, the former having an active surface of such dimension that its diffraction pattern is of the order of centimeters from 1st to 2nd order peaks,

- 23 -

(b) introducing a fluid containing said macromolecules to the active surface to enable binding of said macromolecules to reagent molecules located on the active surface, and

5   (c) detecting the attachment of said macromolecules to the active surface of the probe by determining changes in the position of the diffraction peaks of the order of millimeters corresponding to changes in the dimension of said active surface of the

10 order of nanometers.

3.   The method of claim 1 or 2 wherein the limited size of said former is effective to concentrate said macromolecules of interest on its surface for detection.

4.   The method of claim 1, 2 or 3 wherein said

15 reagent molecules are DNA molecules of a sequence complementary to the sequence of DNA macromolecules of interest.

5.   The method of claim 1, 2 or 3 wherein said reagent molecules are protein molecules.

20   6.   The method of claim 5 wherein said protein molecules are enzymes adapted to detect co-enzymes or substrates.

7.   The method of claim 5 wherein said protein molecules are antibodies associated with an antigen of

25 interest.

8.   The method of claim 1, 2 or 3 wherein said reagent molecules are RNA molecules of a sequence complementary to the sequence of macromolecules of interest.

- 24 -

9. The method of any one of the above claims wherein the critical dimension of said former is of the order of 0.1 to 20 microns and the macromolecules being detected have dimension of the order of fractions of a
5 nanometer to nanometers.

10. The method of any one of the above claims wherein said former is a wire that includes an intermediate layer with attached reagent molecules.

11. The method of any one of claims 1 through 9
10 wherein said former defines a slit in said probe, and said active surface further includes an intermediate layer with attached reagent molecules at the edges of the slit.

12. The method of any one of claims 1 through 9
15 wherein said probe comprises a 0.1 to 10 micron size sphere former that includes an intermediate layer with attached reagent molecules.

13. The method of any one of claims 1 through 9 wherein said former defines an aperture in said probe and
20 said aperture includes an intermediate layer with attached reagent molecules at the edge of said aperture.

14. The method of any one of claims 10 through 13 wherein said reagent molecules are arranged in a manner to form detection and reference surfaces.

25   15. The method of any one of claims 10 through 13 wherein said detection and reference surfaces enable qualitative detection of said macromolecules.

- 25 -

16. The method of any one of claims 10 through 14 wherein said detection and reference surfaces enable quantitative detection of said macromolecules.

17. The method of any one of claims 1 through 16
5 wherein the light is modified to enhance its interaction with the analyte macromolecules.

18. The method of claim 17 wherein the radiation is parallel and coherent to generate the optimum diffraction pattern.

10   19. The method of claim 35 wherein the radiation making up the diffraction pattern is monochromatic.

20. The method of any one of claims 1 through 16 and claim 32 wherein the macromolecules are modified, (such as staining).

15   21. The method of any one of claims 1 through 16 wherein the probe is of a "positive" shape such as a wire or a sphere, or a "negative" shape such as a slit or a pin-hole having, at least one dimension of a suitably small magnitude so as to create a suitably large
20 diffraction effect and so magnification of surface changes.

22. A method of detection of biological macromolecules of clinical significance for diagnosis of disease utilizing a probe with a suitable reagent
25 attached to an active surface of small dimensions and comprising the steps of presenting a suitably prepared sample to the probe, the presence of analyte molecules in the sample resulting in binding of those molecules to the reagent molecules and producing a small dimensional

- 26 -

change on the active surface and thereafter observing the prove, detecting directly the presence of analyte molecules without the use of complicated labelling procedures as used in some other diagnostic techniques.

5   23. A method of detecting biological macromolecules from a solution, said method comprising the steps of:

(a) providing a probe comprising a former with an active surface having the critical dimension in the size range of 0.1 to 100 micron said active surface including reagent molecules exposed for binding the macromolecules of interest to said active surface;

(b) exposing said active surface to a solution containing said macromolecules until they are attached, the limited size of the surface serving to highly concentrate said macromolecules relative to their concentration in the solution;

(c) providing a label capable of attaching to said macromolecules of interest, said label generating a detectable signal;

(d) detecting said signal using a detector of geometry related to the shape of said active surface; and

(e) analyzing said detected signal to determine the presence of said macromolecules.

25   24. The method of claim 23 wherein said label is a radioactive element.

25. The method of claim 23 wherein said label is a fluorescent element.

26. The method of claim 23 wherein said label is a enzyme element.

27. The method of claim 23 wherein said label is a bioluminescent element.

28. The method of claim 23 wherein said label is a chemiluminescent element.

5   29. The method of claim 23 wherein said label is a electroluminescent element.

30. The method of claim 23 wherein said label is a chromophoric element.

31. The method of claim 23 wherein said label is
10 a electrical conductive element.

32. A system for detection of macromolecules comprising:

a probe comprising former of predetermined small size and selected geometry capable of diffracting light
15 of a selected wavelength to produce a diffraction pattern; said former having an active surface with reagent molecules exposed for binding the macromolecules to said active surface;

a light source adapted to emit light of said
20 selected wavelength, said radiation being directed to and extending along said active surface;

said wavelength of the light and the small size of said former being so related that the macromolecules when bound to said active surface are non-transparent and
25 produce a diffraction pattern change which is detectable and greatly amplified in dimension relative to the size of the macromolecule that produces the change;

a detector arranged to detect at least a portion said diffraction pattern; and

- 28 -

a system control adapted to determine the presence of said macromolecules on the basis of said change of the diffraction pattern.

33. The system of claim 32 wherein the limited
5 size of the active surface of said probe is effective to concentrate said macromolecules of interest on its surface for detection.

34. The system of claim 32 or 33 wherein said reagent molecules are DNA molecules of a sequence
10 complementary to the sequence of DNA macromolecules of interest.

35. The system of claim 32 or 33 wherein said reagent molecules are protein molecules.

36. The system of claim 35 wherein said protein
15 molecules are enzymes adapted to detect substrates or co-enzymes.

37. The system of claim 32 or 33 wherein said reagent molecules are RNA molecules of a sequence complementary to the sequence of macromolecules of
20 interest.

38. The system of claim 35 wherein said protein molecules are antibodies associated to antigen of interest.

39. The system of any one of claims 32 through 38
25 wherein the critical dimension of said former is of the order of 0.1 to 20 microns and the macromolecules being detected have dimension of the order of fractions of a nanometer to nanometers.

40. The system of any one of claims 32 through 39 wherein said former is a wire that includes an intermediate layer with attached reagent molecules.

41. The system of any one of claims 32 through 39
5 wherein said former defines a slit in said probe, and said active surface further includes an intermediate layer with attached reagent molecules at the edges of said slit.

42. The system of any of claims 32 through 39
10 wherein said probe comprises a 0.1 to 20 micron size former sphere that includes an intermediate layer with attached reagent molecules.

43. The system of claims 32 through 39 wherein said former defines an aperture in said probe and said
15 aperture includes an intermediate layer with attached reagent molecules at the edge of said aperture.

44. The system of claims 40, 41, 42, or 43 wherein said reagent molecules are arranged in a manner to form detection and reference surfaces.

20   45. The system of claim 44 wherein said detection and reference surfaces enable qualitative detection of said macromolecules.

46. The system of claim 44 wherein said detection and reference surfaces enable quantitative detection of
25 said macromolecules.

47. A system for the detection of macromolecules including a light source, a probe comprising a former of predetermined dimensions, and a detector, all arranged in

- 30 -

a geometry such that the detector observes the
diffraction pattern of the probe, the former having an
active surface of such dimension that its diffraction
pattern at the detection plane is of the order of
5   centimeters from 1st to 2nd order peaks, whereby changes
on the active surface of the order of nanometers in the
dimension result in movements of the order of millimeters
in the position of the diffraction peaks allowing the
attachment of macromolecules to the active surface of the
10   probe to be detected.

        48.   The system of claim 47 wherein the radiation
is modified to enhance the interaction of the radiation
with the analyte macromolecules.

        49.   The system of claim 48 wherein the radiation
15   is parallel and coherent to generate the optimum
diffraction pattern.

        50.   The system of any one of claims 47 through 49
wherein the radiation making up the diffraction pattern
is monochromatic.
20   51.   The system of claim 47 wherein the
macromolecules are modified.

        52.   The system of any one claims 47 through 51
wherein the probe is of a "positive" shape such as a wire
or a sphere, or a "negative" shape such as a slit or a
25   pin-hole having at least one dimension of a suitably
small magnitude so as to create a suitably large
diffraction effect and so magnification of surface
changes.

        53.   A system for detecting biological
30   macromolecules from a solution comprising:

- 31 -

a probe having an active surface of limited size
in the range of about 1 micron to 10 millimeters said
active surface including reagent molecules exposed for
binding the macromolecules of interest to said active
5 surface;

a device adapted to introduce a solution
containing said macromolecules of interest until they are
attached, the limited size of said active surface serving
to highly concentrate said macromolecules relative to
10 their concentration in the solution; said macromolecules
having a label capable to generate a detectable signal;

a detector of geometry correlated to the shape of
said active surface adapted to detect said signal; and

a system control adapted to analyze said detected
15 signal to determine the presence of said macromolecules.

54. The system of claim 53 wherein said label is
a radioactive element.

55. The system of claim 53 wherein said label is
a fluorescent element.

20   56. The system of claim 53 wherein said label is
a enzyme element.

57. The system of claim 53 wherein said label is
a bioluminescent element.

58. The system of claim 53 wherein said label is
25 a chemiluminescent element.

59. The system of claim 53 wherein said label is
a electroluminescent element.

- 32 -

60. The system of claim 53 wherein said label is a chromophoric element.

61. The system of claim 53 wherein said label is a electrical conductive element.

FIG. 1

FIG. 2 — 28, 26, 24, 22, 30, 20

FIG. 2A — 27, 26, 24, 22, 30, 20

FIG. 3F — 28, 26, 24, 22

FIG. 3G — 28, 26, 24, 22, 29

FIG. 3H — 30, 26, 24, 22, 28

FIG. 3I — 32, 26, 24, 22, 30, 28, 20

FIG. 3A

MASK

25

24

22

FIG. 3B

25

24

22

FIG. 3C

26

24

22

FIG. 3D

MASK

25

26

22 24
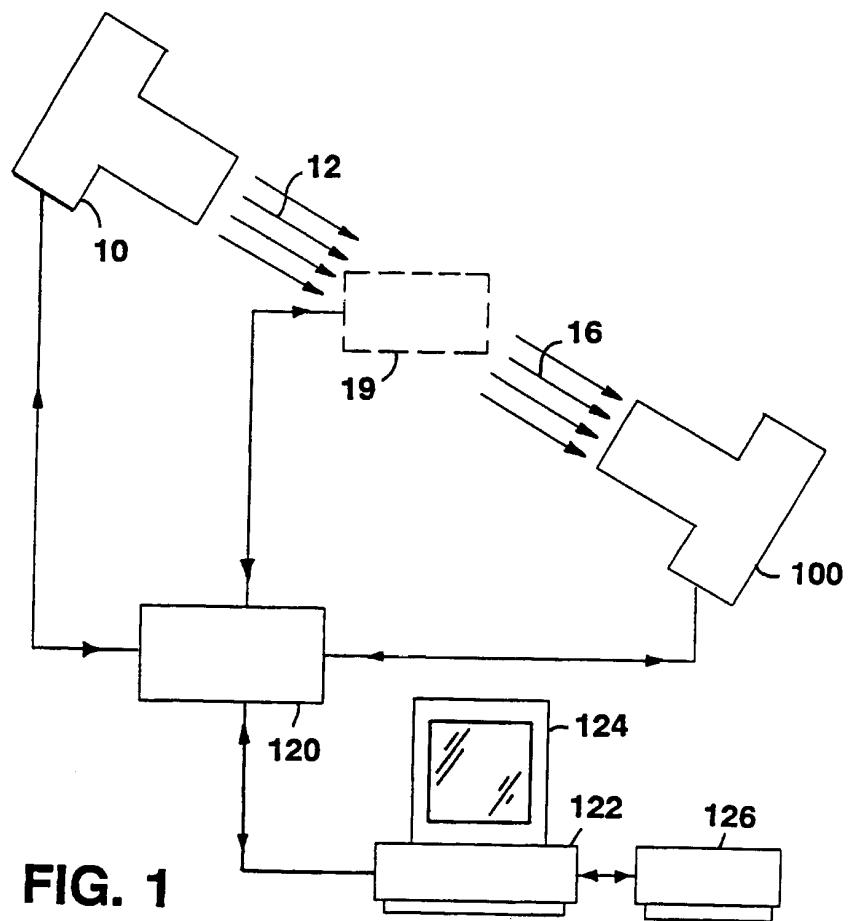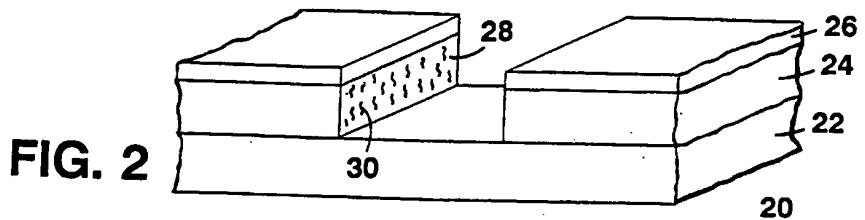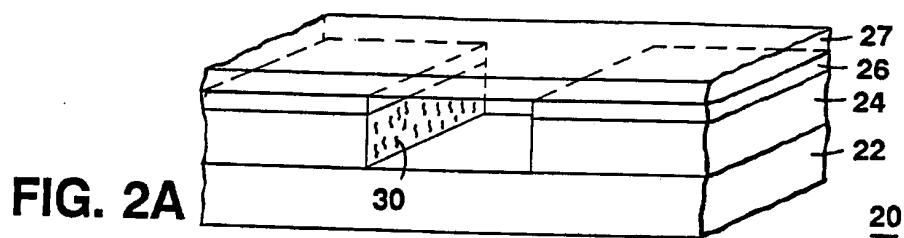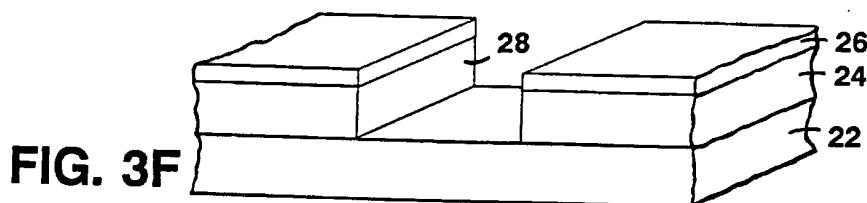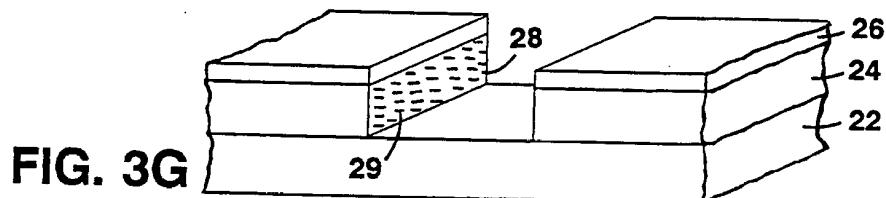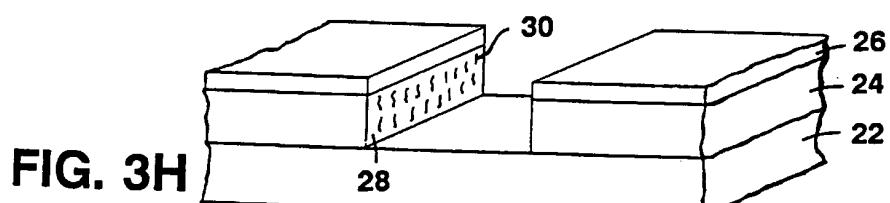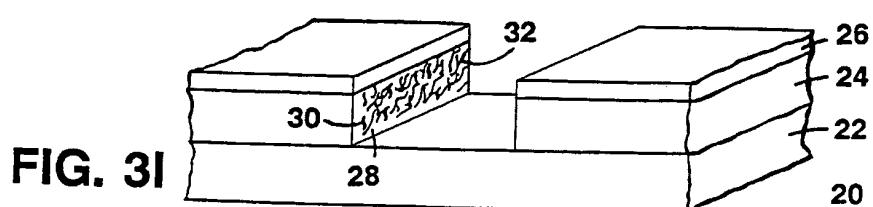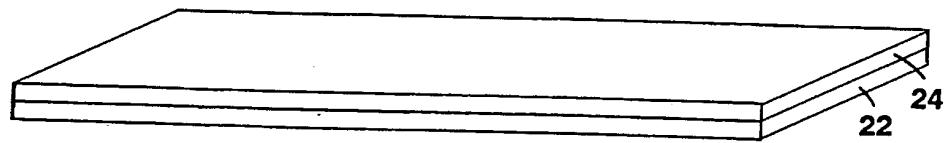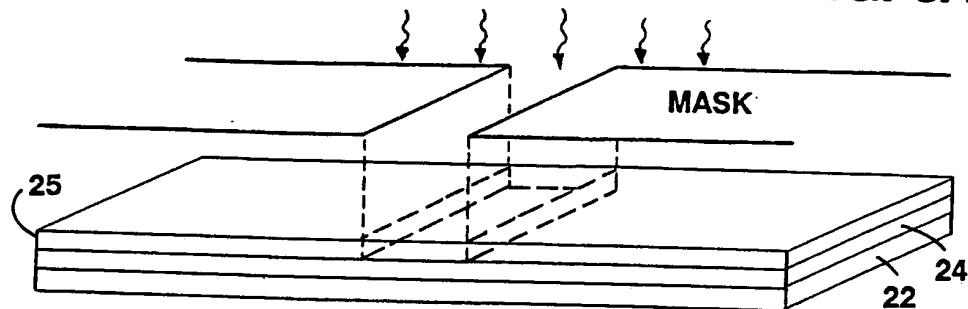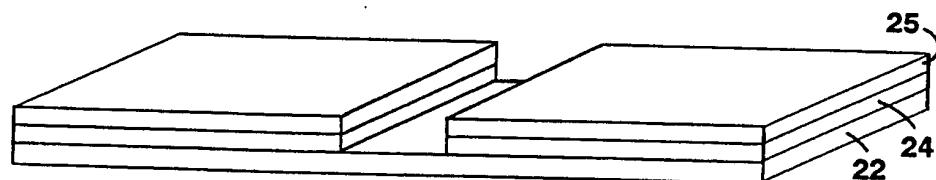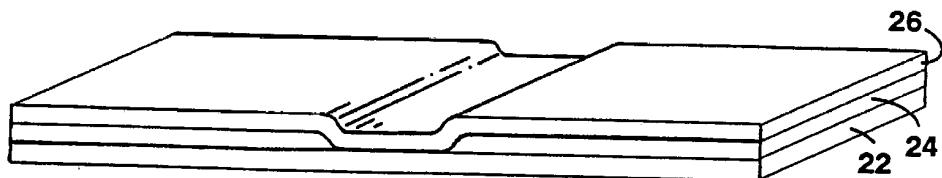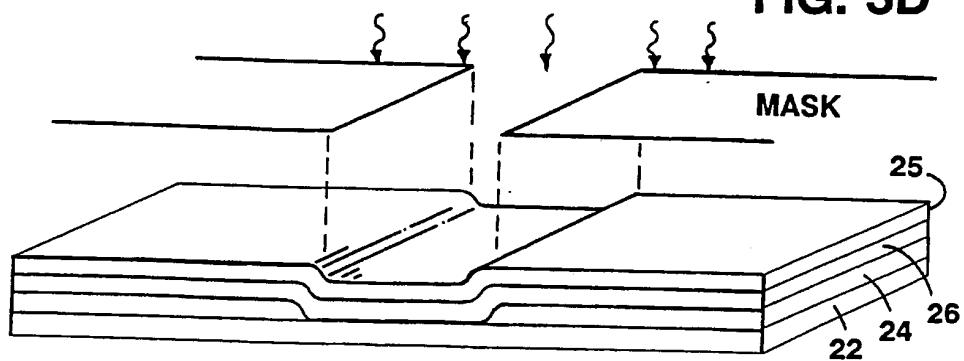
FIG. 3E
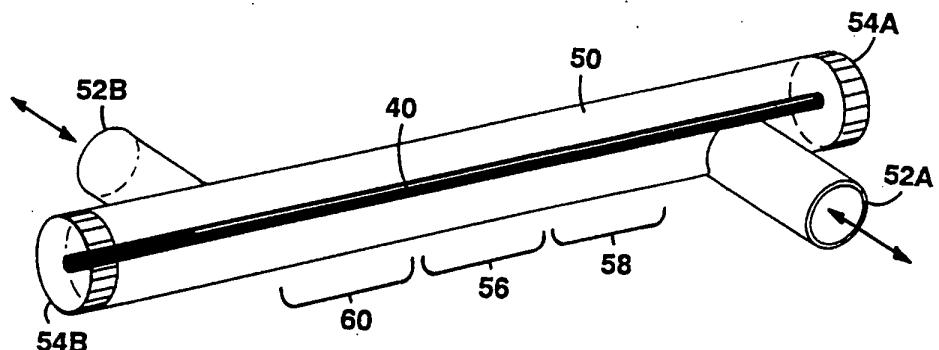
FIG. 4
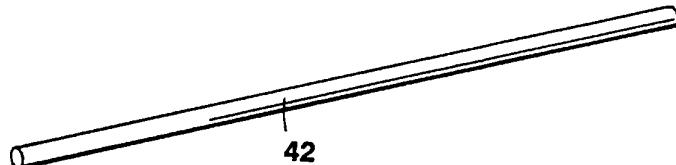
FIG. 5A

FIG. 5B

FIG. 5C

FIG. 5D

FIG. 6

FIG. 6A

FIG. 6B
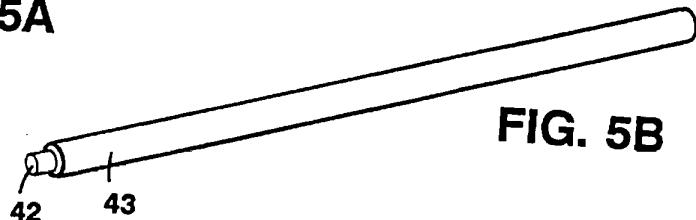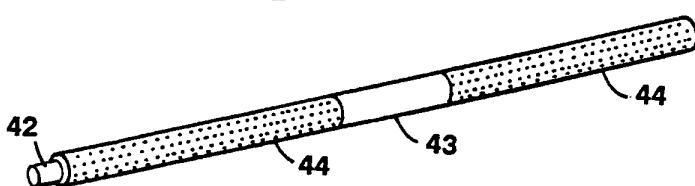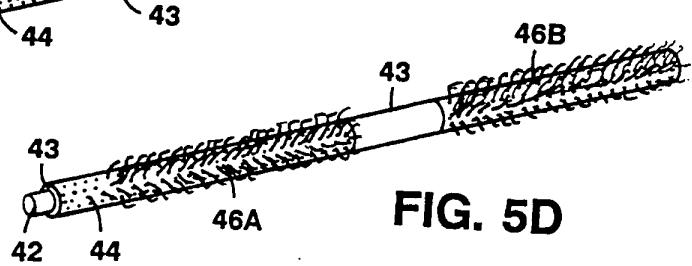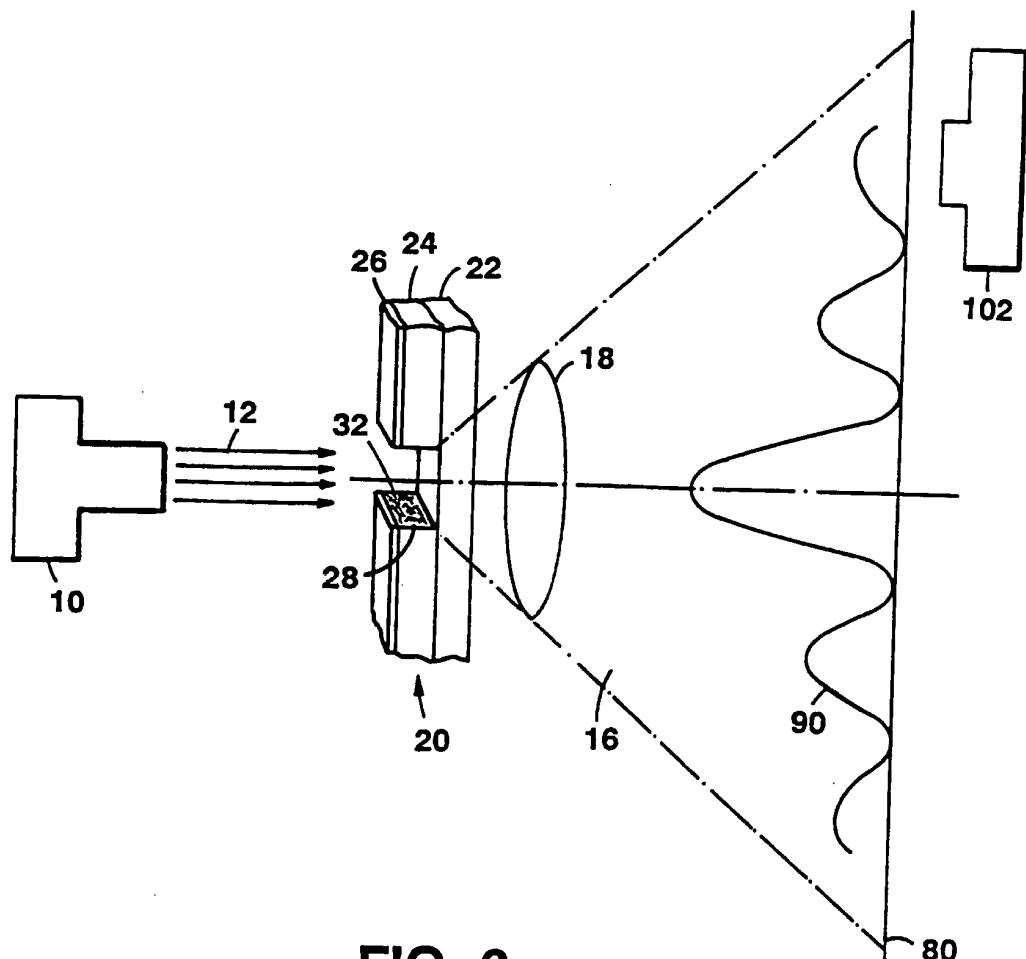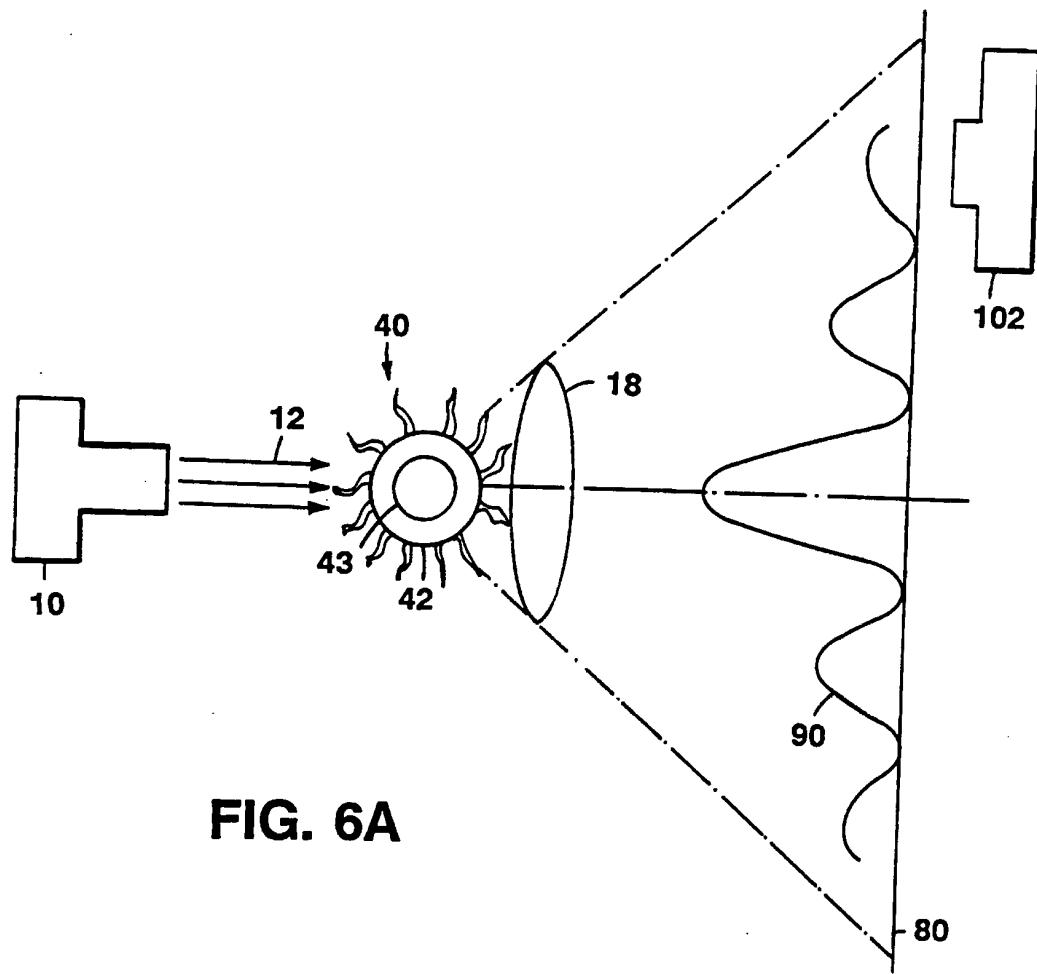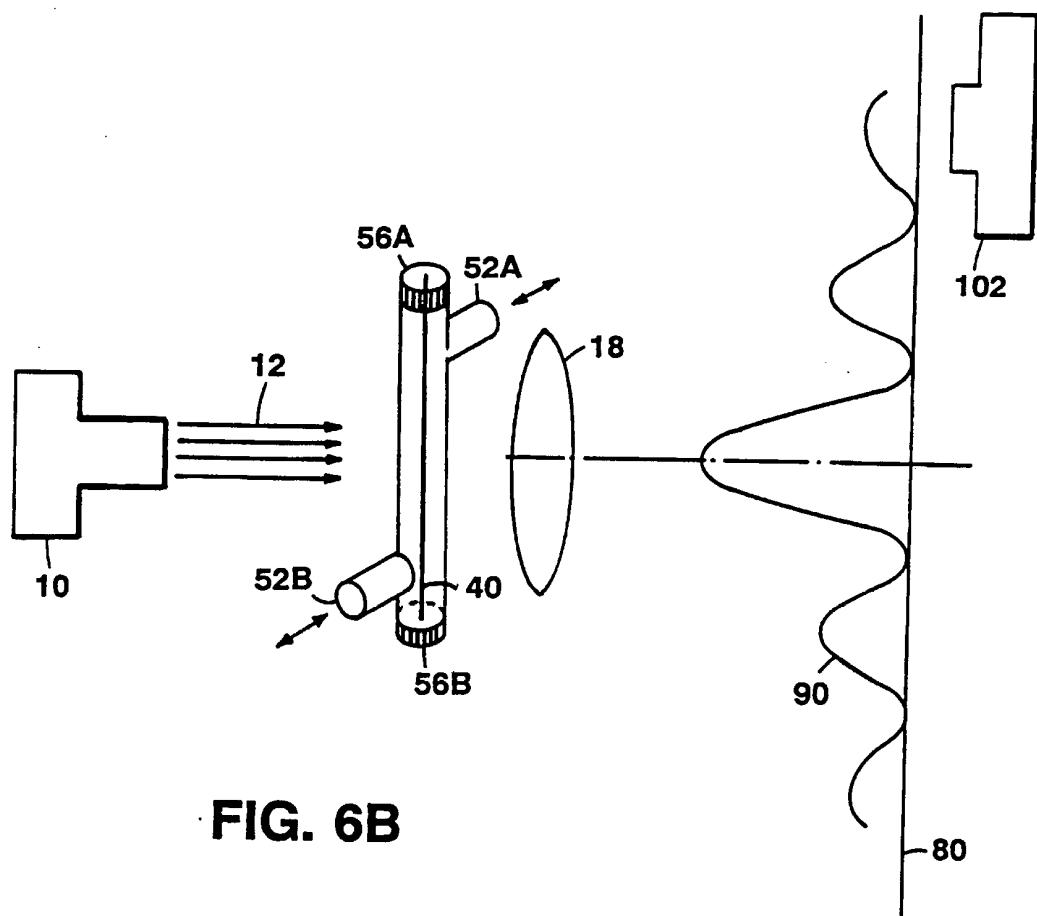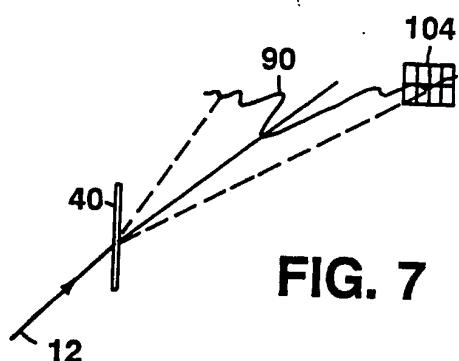
FIG. 7
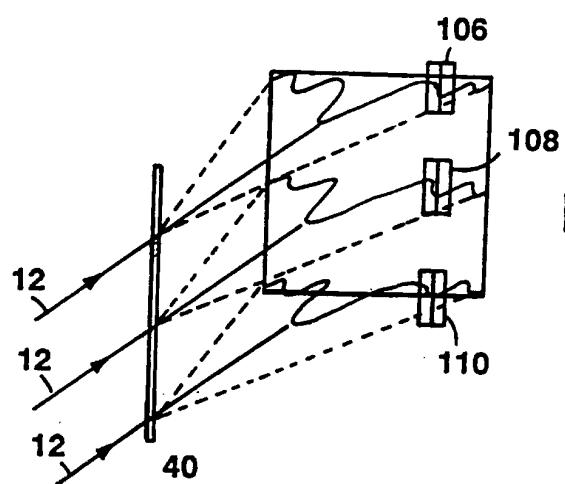
FIG. 8
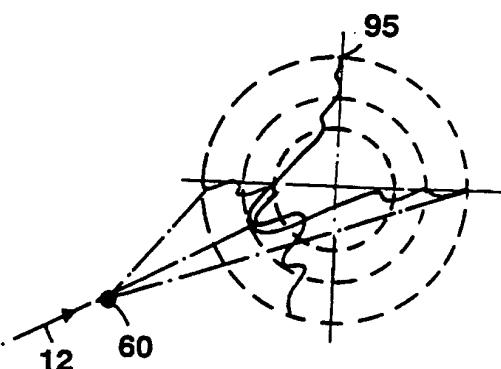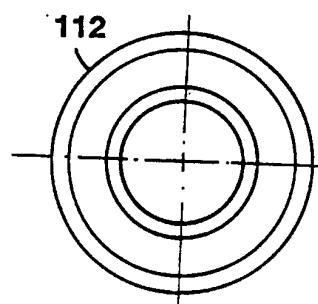
FIG. 9

FIG. 9A
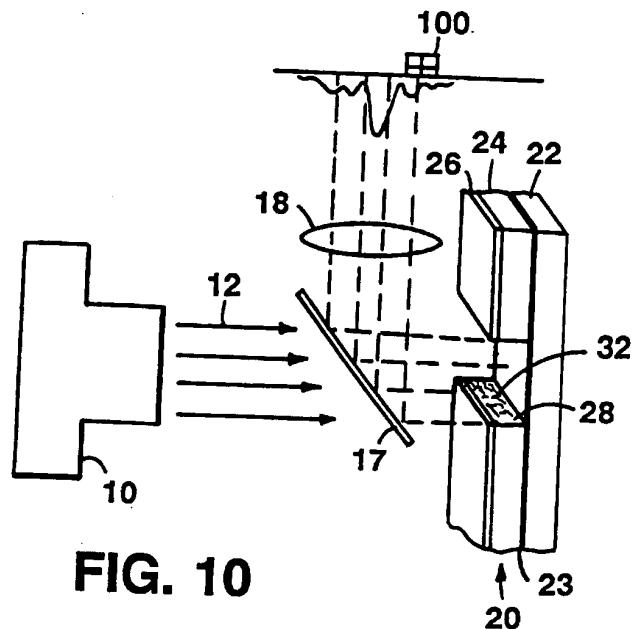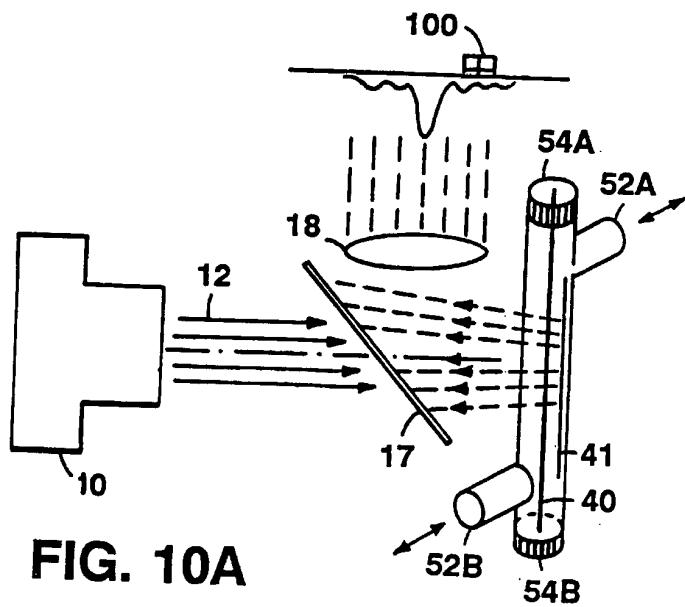
FIG. 10

FIG. 10A

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q 1/68, 1/00; G01N 33/543
US CL :435/6, 7.92, 7.93, 7.94, 7.95; 436/501, 518

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.92, 7.93, 7.94, 7.95; 436/501

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P ___ Y,P	US, A, 5,196,350, (BACKMAN ET AL) 23 MARCH 1993, See the entire document.	1-3, 5, 7, 22, 32, 33, 35, 38, 47-53 ___ 4, 6, 8-21, 23-31, 34, 36, 37, 39-46, 54-61
Y	US, A, 4,876,208, (GUSTAFSON ET AL) 24 OCTOBER 1989, See the entire document.	1-61
Y	US, A, 4,992,385 (GODFREY) 12 FEBRUARY 1991, See the entire document.	1-61
Y	US, A 5,089,387 (TSAY ET AL) 18 FEBRUARY 1992, See the entire document.	1-61

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
02 March 1994	MAR 17 1994

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer
Facsimile No. NOT APPLICABLE	DAVID R. PRESTON *Jell Warden for*
	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*